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(54) **New peptide and gene coding for same.**

(57) Disclosed is a DNA fragment comprising a base sequence coding for a peptide occurring in human atrium cordis and having diuretic action or a precursor of the peptide, plasmids containing the DNA fragment, micro-organisms transformed with the plasmid, and a process for production of the peptide using the transformant.

Also disclosed is a new peptide consisting of 126 amino acids occurring in human atrium cordis and a precursor thereof. The peptide has notable diuretic action and hypotensive or antihypertensive actions.

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                                                  10                  20
Asn Pro Met Tyr Asn Ala Val Ser Asn Ala Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
                                                  30                  40
His Leu Glu Glu Lys Met Pro Leu Glu Asp Glu Val Val Pro Pro Gln Val Leu Ser Glu
                                                  50                  60
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
                                                  70                  80
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly Pro Try Asp Ser Ser
                                                  90                  100
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu
                                                  110                 120
Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
                                                  126
Cys Asn Ser Phe Arg Tyr
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NEW PEPTIDE AND GENE CODING FOR SAME

The present invention relates to a novel peptide, a gene coding for the peptide, a process for production of the peptide using the gene inserted to an expression vector, and use of the peptide.

A normal regulation of the blood pressure in a human body is important for the maintenance of personal health, and various physical and humoral factors contribute to this regulation of the blood vessels, etc. The humoral factors include, for example, the renin-nangiotensin-aldosterone system, catecholamines, prostaglandins, kinin-kallikrein system, and natriuretic hormones including ouabain-like substances. Herein the term "natriuretic" will denote selective excretion of sodium cation relating to potassium cation.

Granules morphologycally similar to granules present in peptide hormone-producing cells are found in human atrium (J.D. Jamieson and G.E. Palade, J. Cell Biol., 23, 151, 1964). An extract of rat atrium and granules contained therein are known to show natriuretic action in rats (A.J. DeBold et. al., Life Science, 28, 89, 1981; R. Keeller, Can. J. Physiol. Pharmacol., 60, 1078, 1982). Recently Mark G., Currie S. et. al. suggested peptide-like substances with a molecular weight of 20,000 to 30,000, or not more than 10,000, present in the atrium of humans, rabbits, swine, and rats, and having natriuretic action (Science, 221, 71-73, 1983).

Moreover, a peptide consisting of 28 amino acid derived from rat atrium cordis was identified (Biochem. Biophys. Res. Commun.; vol 117, No. 3, P 859-865, 1983). The present inventors found a new peptide consisting of 28 amino acids from human atrium

cordis, referred to as "α-human atrial natriuretic polypeptide" and abbreviated as "α-hANP" (Biochem. Biophys. Res. Commun. Vol 118, No. 1, P 131-139, 1984, U.S.P. Serial No. 685151 of the present inventors. The α-hANP has following amino acid sequence:

1 10
H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-
20
Asp-Arg-Ile-Gly-Ala-Glu-Ser-Gly-Leu-Gly-Cys-Asn-
28
Ser-Phe-Arg-Tyr-OH

wherein Cys at the 7 position and Cys at the 23 position are bonded through a disulfide bond.

Various kinds of physiologically active peptides are known to be derived in vivo from their precursors by the action of an endopeptidase or exopeptidase, rather than produced directly. For example, β-endorphin and γ-lipotropin are derived from their precursor β-lipotropin, and β-melanocyte-stimulating hormone is derived from its precursor γ-lipotropin (Takahashi H. et. al., FEBS Lett. 135, 97-102, 1981).

On the basis of the above-mentioned findings, the present inventors expected that the above-mentioned α-hANP would be derived from its precursor, and actually found a new peptide derived from human atrium cordis consisting of 126 amino acids including 28 amino acids of the α-hANP. Moreover, the present inventors successfully obtained a gene coding for the new peptide, and constructed plasmid containing the gene which expresses in a microorganism.

Therefore, the present invention provides a new peptide consisting of 126 amino acids and having diuretic action and hypotensive or antihypertensive action. The peptide is hereinafter referred to as "γ-human atrial natriuretic polypeptide" and abbreviated as γ-hANP".

There is also provided a DNA fragment comprising a base sequence coding for a peptide occurring in human

atrium cordis and having diuretic action or a precursor of the peptide.

There is also provided plasmids containing a promotor region, SD sequence, and the above-mentioned DNA fragment, and capable of producing a peptide coded by the DNA fragment in a microoganism.

There is also provided a microorganism transformed with the plasmid.

The present invention further provides a process for the production of the γ-hANP or precursor peptide thereof by culturing the transformed microorganism in a culture medium and recovering the γ-hANP or precursor from the cultured cells or medium, or converting either such peptide to or from an acid addition salt.

The present invention further extends to provide a pharmaceutical composition containing the γ-hANP as a diuretic or hypotensor and extends to medical use of γ-hANP.

The invention will be further explained and embodiments and examples set forth, in the description below, referring to the drawings in which:

Figure 1 represents an amino acid sequence of γ-hANP consisting of 126 amino acids;

Figure 2 represents an amino acid sequence of a precursor of the γ-hANP, which sequence consists of 151 amino acids wherein a sequence of from 26 to 151 corresponds to the sequence of the γ-hANP set forth in Figure 1;

Figure 3 represents a base sequence coding for the amino acid sequence set forth in Figure 1;

Figure 4 represents a base sequence coding for the amino acid sequence set forth in Figure 2;

Figure 5 represents a sequence of a cDNA fragment cloned into plasmid phANP82 including a 5' non-coding region, a coding region corresponding to the base sequence set forth in Figure 4, and a 3' non-coding region;

Figure 6 represents a construction process of

plasmid pS220 from a plasmid pS20 and a DNA fragment containing a base sequence coding for amino acid sequence of γ-hANP;

Figure 7 represents a result of electrophoresis showing expression of a γ-hANP gene.

Figure 8 represents a construction process of plasmid pS223-3 from plasmid pS83-3 and a DNA fragment containing a base sequence coding for amino acid sequence of γ-hANP, and a construction process of plasmid pS224-3 from plasmid pS84-3 and the same DNA fragment;

Figure 9 represents base sequences of probes used for selection of DNA fragments coding for amino acid sequence of γ-hANP;

Figure 10 represents a strategy used for sequencing DNAs of plasmid phANP1 and phANP82;

Figure 11 represents graphs showing diuretic action of γ-hANP;

Figure 12 represents a chart showing the hypotensive action of the γ-hANP;

Figure 13 represents a chromatogram showing an elution profile using Sephadex G-75 during the purification of the γ-hANP;

Figure 14 represents an elution profile of a high performance liquid chromatography (HPLC) during the purification of the γ-hANP; and

Figure 15 represents an elution profile of HPLC of purified γ-hANP.

1. Identification of γ-hANP Gene

RNA were extracted from human artrium cordis by a method disclosed in Chirgwin (Chirgwin, J. M. et. al., Biochemistry 18, 5294-5299, 1979), and enriched for poly $(A)^+$ RNA (mRNA) with an oligo (dT) cellulose column. The poly $(A)^+$ RNA were used to prepare a cDNA library according to the Okayama-Berg method (Mol. Cell. Biol. 2, 161-170, 1982). The library was screened with a mixture of probes consisting of synthesized 14 mer-

oligonucleotides labeled with ^{32}P coding for an amino acid sequence of from 12 to 14 of α-hANP (Fig. 9), and 23 clones were selected which hybridized with the probes. Among the 23 clones, 8 clones were used to extract plasmids which were then sequenced with the mixture of probes by the dideoxy method. As a result, all of the plasmids tested contained a base sequence coding for α-hANP. Among these plasmids, two plasmids containing a longer insert DNA fragment, i.e., plasmids phANP1 and phANP82, having about 980 base pairs (pb) and about 850 pb, respectively, were selected, and the insert fragments were completely sequenced by the Maxam and Gilbert method (Meth. Enzym., 65, 499-560, 1980) and the method disclosed by Sanger et. al. (Proc. Natl. Acad. Sci. USA, 74, 5463-5467, 1977). Both fragments had a long open reading frame which starts from a translation initiation codon ATG and ends at a translation stop codon TGA. However, the fragment in the plasmid phANP82 was shorter by about 100 bp than the fragment in the plasmid phANP1. A base sequence of the fragment in the plasmid phANP82 is shown in Fig. 5. Figure 5 also contains an amino acid sequence expected from the base sequence of the open reading frame. The expected amino acid sequence consists of 151 amino acids and includes an amino acid sequence of α-hANP already established, which sequence is shown in a box in Fig. 5.

On the other hand, a peptide having a molecular weight of about 13,000 Dalton was isolated from human atrium cordis. The peptide was sequenced, and determined to have an amino acid sequence set forth in Fig. 1 (described below in detail).

The amino acid sequence of the peptide extracted from human atrium cordis is identical with an expected amino acid sequence from the 26 position to 151 position in Fig. 5. This means that the cDNA fragment in the plasmid phANP82 contains a base sequence coding for γ-hANP. the cDNA fragment also contains an additional

base sequence coding for an amino acid sequence from Met at position 1 to Ala at position 25 in Fig. 5. The amino acid sequence from 1 to 25 is believed to be a signal peptide which participates in the secretion of a peptide. As shown above and in Fig. 5, the cDNA region in the plasmid phANP82 consists of a 5' non-translation region of 90 bp, a coding region of 453 bp, and a 3' non-translation region of 300 bp. The coding region consists of a region coding for a signal sequence of from 1 to 25 in amino acid position and a region coding for γ-hANP peptide of from 26 to 151 in amino acid position. The region coding for γ-hANP peptide includes a region coding for α-hANP peptide of from 124 to 151 in amino acid position.

The above-mentioned relationship between α-hANP and γ-hANP supports the expectation that γ-hANP is a precursor of α-hANP. When α-hANP is derived from γ-hANP, an amide bond of the Pro-Arg sequence present at 122 and 123 in Fig. 5, i.e., an amide bond between Agr-Ser at 123 and 124, has to be cleaved. This possibility is strongly suggested from a phenomenon wherein gastrin releasing peptide is derived from its precursor by cleavage of an amide bond of the Pro-Arg sequence present in the precursor (Reave, J.R. et. al., J. Biol. Chem. 258, 5582-5588, 1983; and Minamino, N. et. al., Biochem. Biophys. Res. Commun. 119, 14-20, 1984). From the similarity between γ-hANP and gastrin releasing peptide in the presence of the Pro-Arg sequence, α-hANP is reasonably considered to be formed in vivo from γ-hANP by processing (perhaps by enzymic action).

2. Construction of Recombinant DNA

As DNA fragments cloned into plasmide phANP1 and phANP82 contain a base sequence coding for γ-hANP, and therefore α-hANP, both of which have natriuretic or diuretic action and hypotensive or antihypertensive action, the DNA fragments are useful for the industrial production of γ-hANP and α-hANP and other diuretic

peptides having an amino acid sequence present in the γ-hANP.

For the construction of a recombinant DNA containing a base sequence coding for γ-hANP and useful for expression of the γ-hANP gene, a restriction enzyme EcoRI cleavage site and a translation initiation codon ATG were incorporated into an upstream site adjacent to the γ-hANP gene in the DNA fragment by in vitro mutation (Zoller, M. J. & Smith, M., Nucl. Acid. Res. 10, 6487, 1982). RF-DNA of M13mp8 and DNA of plasmid phANP1 were cleaved with Pst I to obtain cleaved RF-DNA of M13mp8 and a DNA fragment of about 700 bp containing the γ-hANP gene, respectively. Both DNA fragements were ligated with T4 DNA ligase, and transformed into E. coli JM103. The transformed E. coli was cultured to obtain a single strand phage containing the 700 bp fragment. The single strand phage DNA was used to from a hetero duplex with a chemically synthesized 36 mer single strand DNA. The synthesized DNA consists of a base sequence GAATTCATG coding for the above-mentioned EcoRI site and the translation initiation codon ATG and corresponding to a sequence ACC AGA GCT in Fig. 5 (shown by a dotted line), a base sequence of 12 bases identical with a sequence upstream of the dot-lined sequence, and a base sequence of 15 bases identical with a sequence downstream of the dot-lined sequence. The single strand DNA partially having the hetero duplex was converted to a double strand DNA according to a conventional method with a DNA polymerase I Klenom fragment (Zoller, J.M. & Smith, M., Nucl. Acid Res. 10, 6487, 1982). The formed double strand DNA was transformed into E. coli JM103. To select plasmid DNA containing a base sequence GAATTCATG in place of the original base sequence ACCAGAGCT, screening was carried out by culturing the transformed E. coli, obtaining DNAs, and cleaving the DNAs with EcoRI. A base sequence covering the sequence GAA TTC ATG was determined by a dideoxy chain termination method

(Sanger, F. et. al., Proc. Natl. Acid. Sci. USA 74, 5463-5467, 1977), and a plasmid containing a desired sequence was selected and designated as M13mp8-hANP525.

To construct an expression plasmid, the plasmid M13mp8-hANP525 was cleaved with Eco RI and Sal I to obtain a DNA fragment of about 510 bp containing the γ-hANP gene. The Eco RI cleaves the Eco RI cleavage site incorporated by the above-mentioned in vitro mutation; and the Sal I cleaves a Sal I cleavage site originally present in M13mp8. A plasmid pS20 (Fig. 6) was cleaved with Eco RI, and Sal I, and a Eco RI - Sal I DNA fragment of about 450 bp was replaced with the above-mentioned Eco RI - Sal I fragment of about 510 bp to obtain an expression plasmid pS220 (Fig. 6). The plasmid pS20 has a λP_L promotor and SD sequence of a λ phage CII protein gene as a non-translation region followed by an Eco RI cleavage site, and can express foreign gene inserted at the Eco RI under the control of the λP_L promotor. The plasmid pS20 has been constructed by the inventor, and deposited at the Fermentation Research Institute Agency of Industrial Science and Technology (FRI) under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent (Budapest Treaty) as FERM BP-535 on May 30, 1984.

The plasmid pS220 was transformed into E. coli N4830, and the transformed strain was cultured in a minimum medium M9 supplemented with 50 μg/ml ampicillin and 19 natural amino acids except for L-methionine at a temperature of 32°C. When the E. coli was grown to OD_{660} = 0.4, the temperature was shifted from 32°C to 42°C, and ^{35}S-methionine was added to the culture to pulse-label the newly synthesized protein for two minutes. Culturing was continued for an additional 15 minutes or 30 minutes, and terminated by the addition of trichloroacetic acid. A control culture was prepared with E. coli N4830 not containing plasmid pS220. Pro-

teins were extracted and analysed with SDS-polyacrylamido gel lectrophoresis (SDS-PAGE) to determine the composition of the proteins. the culture of E. coli containing plasmid pS220 provided a band of about 20Kd which was not found in the control culture (Fig. 7). This means that the plasmid pS220 expressed a new protein with about 20Kd.

Moreover, plasmid pS223-3 and pS224-3 were constructed. The plasmid pS223-3 was constructed from plasmid pS83-3 and an Eco RI - Sal I DNA fragment of about 510 bp containing the γ-hANP gene from the M13mp8-hANP525. The plasmid pS223-3 contains an SD sequence of the lpp gene in place of the region flanked by the λP_L promotor and a translation initiation codon ATG in plasmid pS220 (Fig. 8). The plasmid pS224-3 was constructed from plasmid pS84-3 and the above-mentioned 510 bp DNA fragment. The plasmid pS224-3 contains an SD sequence of the MS2 phage A protein in place of the region flanked by the λP_L promotor and the ATC (Fig. 8). Both E. coli transformed with pS223-3 and E. coli transformed with pS224-3 produced the same proteins as those produced by E. coli transformed with pS220.

While the molecular weight (about 20Kd) shown on the SDS-PAGE is higher than a theoretical molecular weight of γ-hANP (13,679 Dalton), the protein showing about 20Kd was determined to be γ-hANP because γ-hANP prepared from human atrium cordis migrates to a site corresponding to 20Kd on the SDS-PAGE, and because the SDS-PAGE often shows a molecular weight higher than a theoretical molecular weight for other proteins.

This conclusion was confirmed as follows: a chemically synthesized linker,

5' - CTAAGTAAGTAAG
GATTCATTCATTCAGCT

wherein the underlined portions are translation termination codons, and each of the codons corresponds to any one of three kinds of reading frames, was incorporated

into a site downstream of the γ-hANP gene in a expression plasmid. The E. coli transformed with the plasmid having the three additional termination codons again produced a protein having a 20Kd molecular weight on the SDS-PAGE. This result negates the possibility that the translation termination codon TGA present at the next position to the 151st codon TAC in Fig. 5 was translated.

Thus, it was confirmed that γ-hANP, which is a precursor of α-hANP, can be produced by a microorganism using a recombinant gene according to the present invention.

Wherein, although construction of plasmids containing a base sequence coding for an amino acid sequence from 26 to 151 in Fig. 5 (γ-hANP) as a coding region are represented, other plasmids containing a base sequence coding for an amino acid sequence from 1 to 151 in Fig. 5 (signal sequence and γ-hANP), and plasmids containing a base sequence coding for an amino acid sequence from 124 to 151 (α-hANP) or any other amino acid sequence as a coding region can be constructed by the same procedure. Moreover, although cDNA corresponding to mRNA prepared from human atrium cordis was represented as a γ-hANP gene, gene coding for γ-hANP can be chemically synthesized. Moreover, although the λP_L promotor was represented as a promotor region in expression plasmids, other promotors can be used. For example, to express the gene in E. coli, a promotor region of tryptophan gene (trp), lactose gene (lac), lipoprotein gene (lpp), alkaline phosphatase genes (PHOA, PHOS, etc.) or outer membrance protein gene (omp), or a hybrid promotor of tryptophan and lactose promotors (tac promotor) can be used. To express the gene in yeast, a promotor region of alcohol dehydrogenase gene (ACH), glyceraldehyde dehydrogenase gen (GAP-DH), phosphoglycerokinase gene (RGK), or repressible acid phosphatose gene can be used. Moreover, to express the gene in animal cells, or a promotor region of SV40, an early or

late gene can be used.

3. Preparation of γ-hANP from Human Atrium Cordis

γ-hANP can be produced by the extraction of the γ-hANP from human atrium.

In the process, human atrium is homogenized in an acidic aqueous solution such as a phosphate buffer solution, or an acetic acid solution containing hydrochloric acid. Subsequently, γ-hANP is purified according to a conventional method suitable for the purification of peptide, such as centrifugation, isoelectric point precipitation, solvent extraction, ultrafiltration, gel filtration, adsorption chromatography or high performance liquid chromatography (HPLC), or a combination of such methods. In the above-mentioned methods, chicken rectum relaxation activity is conveniently used to select fractions containing γ-hANP, because γ-hANP has this activity. In the chromatography methods, the α-hANP containing fractions can be also selected by molecular weight (about 14,000).

4. Structure and Physico-chemical Properties of γ-hANP

1) Structure:

The γ-hANP has the structure set forth in Fig. 1.

2) Molecular weight: about 13,000 as determined by gel-filtration and about 20,000 as determined by SDS-PAGE (13679 as calculated).

3) UV spectrum: Max = 276 mm.

4) Color reactions: Ehrlich's reaction, negative; Sakaguchi's reaction, positive; Pauly's reaction, positive.

5) Distinction of basic, acidic or neutral property: basic.

6) Solubility in solvents: soluble in water, methanol, and acetic acid; insoluble in ethyl acetate, butyl acetate, ethyl ether, hexane, petroleum ether, benzene, and chloroform.

7) Amino acid composition by amino acid analysis:

Amino acid	Mol. ratio	
	found	calculated
Asx	13.69	14
Ala	11.98	11
Arg	10.24	10
Ile	1.45	1
Gly	11.61	11
Glx	13.22	12
$(Cys)_2$	0.16	1
Ser	12.37	13
Tyr	2.01	2
Phe	3.48	3
Met	3.61	4
Leu	17.22	16
Val	5.98	6
Thr	2.68	2
His	1.43	1
Lys	5.14	4

8) Formation of salts: the γ-hANP is a basic compound as described in item 5), and can form acid addition salts with an inorganic acid such as hydrochloric acid, sulfuric acid, phosphoric acid, or an organic acid such as formic acid, acetic acid, propionic acid, succinic acid, and citric acid.

5. Physiological Properties of γ-hANP

The γ-hANP according to the present invention has notable diuretic, and hypotensive or antihypertensive actions.

Test method:

Male rats weighing 300 to 400 grams were anesthetized by intraperitoneal administration of

pentobarbital at a dosage of 60 mg/kg, and used for tests on the γ-hANP according to the method described in Life Sciences, Vol. 28, pp89-94, 1981, A.J. deBold, et·al.

To keep the respiratory tract open, a tracheal cannula (PE-240 Clay-Adams) was inserted into the trachea. An arterial cannula (PE-50) was inserted into a femoral artery for measurement of the blood pressure, and a venous cannula was inserted into a femoral vein for the administration of Ringer's solution. 1.2 ml of Ringer's solution was injected for ten minutes, and subsequently, was constantly infused at a flow rate of 1.2 ml/hour.

A bladder cannula made of silastic tube with a inner diameter of 0.02 inches and an outer diameter of 0.037 inches was inserted into the bladder, and via the cannula, a urine sample was collected into a test tube. The collection of urine was carried out for 30 minutes before administration of the test compound, and 5, 10, 20, 30, and 60 minutes after the administration.

1 nmole of the test compound γ-hANP was dissolved in 50 µl of sterilized physiological saline with 5 µg of bacitracin, and the solution was injected into the jugular vein.

As shown in Fig. 11, γ-hANP shows a notable diuretic action. Moreover, as shown in Fig. 12, after 1 mole of γ-hANP is administered to a rat, the blood pressure is gradually lowered by about 20 mmHg for about 1 hour, revealing that γ-hANP has a hypotensive action or antihypertensive action, and may be useful as a hypotensor.

6. Use of γ-hANP as a pharmaceutical product

Repeated administration of γ-hANP does not stimulate production of antibodies, and does not cause anaphylaxis shock. γ-hANP consisting of L-amino acids is gradually hydrolized in a body providing the L-amino

acids, and therefore shows little toxicity.

Due to the higher diuretic, and blood pressure-lowering or antihypertensive actions, and the lower toxicity, γ-hANP is useful as an active ingredient for pharmaceutical compositions such as a diuretic and a hypotensor. γ-hANP is administered at 1 μg/kg to 10 mg/kg, preferably 10 μg/kg to 1 mg/kg.

γ-hANP can be administered in the same manner as conventional peptide type pharmaceuticals. Namely, γ-hANP is preferably administered parenterally, for example, intravenously, intramuscularly, intraperitoneally, or subcutaneously. γ-hANP, when administered orally, may be proteolytically hydrolyzed. Therefore, oral application is not usually effective. However, γ-hANP can be administered orally as a formulation wherein γ-hANP is not easily hydrolyzed in a digestive tract, such as liposome-microcapsules. γ-hANP may be also administered in suppositorys, sublingual tablets, or intranasal spray.

The parenterally administered pharmaceutical composition is an aqueous solution containing about 0.000005 to 5%, preferably 0.00005 to 0.5% of γ-hANP, which may contain, in addition to γ-hANP as an active ingredient, for example, buffers such as phosphate, acetate, etc., osmotic pressure-adjusting agents such as sodium chloride, sucrose, and sorbitol, etc., antioxidative or antioxygenic agents, such as ascorbic acid or tochopherol, and preservatives, such as antibiotics. The parenterally administered composition also may be a solution readily usable or in a lyophilized form which is dissolved in sterile water before administration.

Examples

The present invention will now be further illustrated by, but is by no means limited to, the following examples.

Example 1.

1) Preparation of cDNA library, and isolation and identification of α-hANP gene

(1-a) Preparation of cDNA library

From two human atrium cordis obtained from an 82 years old female and 61 years old male, 1 mg of RNA was extracted with 4M guanidium thiocyanate according to a method of Chirgwin et al. (Chirgwin, J.M. et. al., Biochemistry 18, 5294-5299, 1979). The RNA was then subjected to an oligo (dT) cellulose column using 10 mM Tris-HCl buffer, pH 7.2, containing 0.5M LiCl, 10 mM EDTA and 0.5% SDS as a binding buffer, and 75 μg of poly $(A)^+$ RNA (mRNA) was isolated (Nakazato, H. & Edmonds, D.S., Meth. Enzym. 29, 431-443, 1974). 15 μg of the poly $(A)^+$RNA and 4.2 μg of vector primer DNA were used to prepare a cDNA library (plasmids) according to the Okayama-Berg method (Mol. Cell Biol. 2, 161-170, 198), and the cDNA library was used to transform E. coli WA802. The transformants were screened on an LB-agar medium supplemented with 40 γ/ml of ampicillin, and about 40,000 colonies of ampicillin resistant transformants were obtained per microgram of starting mRNA.

(1-b) Isolation of α-hANP clone

About 40,000 colonies were replicated on a nitrocellulose filter, and the filter was incubated on an LB agar plate supplemented with 40 γ/ml of ampicillin at 37°C for 6 hours. The filter was transferred onto an LB ager plate supplemented with 180 γ/ml of chloramphenicol, and incubated at 37°C over night. The colonies on the filter were lysated with 0.5N NaOH, and neutralized to pH 7.0, and the filter was soaked in 0.5 M Tris -HCl buffer, pH 7.0, containing 1.5 N NaCl, and in 3 x SCC (0.15 M NaCl, 0.05M sodium citrate) for 5 minutes respectively. Finally, cell debris on the filter was removed with a paper towel, and the filter was air-dried and then baked at 80°C for 2 hours. The filters were then subjected to hybridization with a mixture of probes I and II (Fig. 9) consisting of

chemically synthesized 14-mer oligonucleotides labeled with ^{32}p at their 5'-end (Grunstein, M. & Hogness, D.S., Proc. Natl. Acad. Sci. USA, 72, 3961-3965, 1975). The 14-mer oligonucleotides used as a probe are possibly complemental with mRNA coding for an amino acid sequence Met-Asp-Arg-Ile-Gly (an amino acid sequence from 135 to 139 in Fig. 5), and have been labeled with ^{32}p at their 5'-end using ^{32}p γ-ATP and T4 kinase and have a specific activity of 1 to 3 x 10^6 cpm/p mole. The hybridization was carried out in 3 x SCC containing 1 x Denhardts (0.2% BSA, Armour Pharmaceutical Company; 0.2% Ficol, Sigma; and 0.2% polyvinyl pyrrolidone, Wako Jyunyaku), 0.1% SDS and 50 µg/ml salmon testis DNA, at 38°C for 16 hours. The filter was then washed with 3 x SCC containing 0.1% SDS, air-dried, and placed in contact with an X-ray film. As a result, 85 positive clones were observed on the film. The 85 positive clones were then subjected to the colony hybridization using the same procedure as described above, except that the probe I and probe II were separately used at 40°C and 38°C for each probe. As a result, 23 clones were obtained which hybridize with the probe II but do not hybridize with the probe I. Among these 23 clones 8 clones were used to isolate plasmid DNA according to a conventional method. The isolated plasmid DNAs were sequenced using the probe II as the primer according to a dideoxy chain termination method (Sanger F. et al, Proc. Natl. Acad. Sci. USA, 74, 4563-5467, 1977). As a result, all of the plasmids contained a base sequence corresponding to a part of an amino acid sequence of α-hANP and, conseqnently, the above-mentioned 8 clones were confirmed to have a plasmid containing cDNA of α-hANP. Among the 8 plasmids, 2 plasmids having a longer insert containing cDNA of γ-hANP were selected, and designated as phANP 1 and phANP82. The inserts of plasmids phANP1 and phANP82 were sequenced. As a result, the plasmids phANP1 and phANP82 contained an

insert of about 950bP and an insert of about 850bp, respectively.

Restriction enzyme sites of the insert DNA region and a strategy for sequencing the insert DNA region are set forth in Fig. 10. In Fig. 10, the direction for sequencing is shown by arrows, wherein solid lines show an upper strand and dotted lines show a lower strand. The sequencing was carried out according to the Maxam-Gilbert method (Meth. Enzym., 65, 499-560, 1980) and Sanger method (Proc. Natl. Acad. Sci. USA, 74, 5463-5467, 1977). A shows the sequencing according to the Maxam-Gilbert method; B shows the sequencing according to the Sanger method; and C shows the sequencing wherein each plasmid phANP1 and phANP82 was cleaved with PstI and PvuII to obtain 3 fragments, and then each fragment was inserted into plasmid pUC8 (Vieira, J. & Messing, J., Gene 19, 259-268, 1982) and sequenced according to the Sanger method.

As a result of the above-mentioned sequencing, it was comfirmed that the base sequences of inserts present in the plasmids phANP1 and phANP82 were identical except that the insert in the plasmid phANP1 has an additional base sequence of about 100bp as the 5' non-translation region. Figure 5 shows the base sequence of the insert in phANP82 and an amino acid sequence corresponding to an open reading frame in the insert. The insert DNA contains the reading frame starting from ATG and ending at a translation termination codon TGA, and the reading frame includes a base sequence coding for α-hANP at the 3' terminal side (C terminal side in the amino acid sequence). On the basis of an amino acid sequence of γ-hANP purified from human atrium cordis and identified, which has an N terminal sequence H-Asn-Pro-Met-Tyr-Asn- ... and C terminal sequence ... -Asn-Ser-Phe-Arg-Tyr-OH as set forth in Fig. 1, the reading frame was confirmed to contain a coding region of from codon AAT of Asn at 26 to codon ATC of Tyr at

151 in Fig. 5.

In Fig. 5, an amino acid sequence of from Met at 1 to Ala at 25 is considered from its characteristic sequence to be a signal sequence participating in the secretion of peptide. The base sequence in Fig. 5 also contains a sequence AATAAA shown by a solid underlining, which sequence is known to precede polyadenylation site in many eukaryotic mRNA.

2) Construction of γ-hANP gene expression vector

(2-a) Insertion of γ-hANP gene into M13 DNA

0.44 µg of M13mp8 RF-DNA was cleaved with 16 units of PstI in 20 µl of Medium-Salt Buffer (10 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) at 37°C for 1 hour. The mixture was then heated at 65°C for 10 minutes to stop the enzyme reaction. On the other hand, 20 µg of plasmid phANPI DNA was cleaved with 160 units of PstI in 100 µl of the Medium Salt Buffer at 37°C for 1 hour, and the reaction mixture was subjected to 1 % agarose gel electrophoresis. A part of the gel containing an DNA fragment corresponding to about 700bp was cut to obtain an agarose piece, and the DNA fragment was extracted by the electro-elution method and purified.

66 ng of the DNA fragment from M13mp8 RF-DNA and 1 µg of the 700 bp DNA fragment were ligated using 5.6 units of T4 DNA ligase (Takara Shuzo, Japan) in 20 µl of ligation buffer (20 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 1 mM ATP, 5 mM DTT) at 14°C for 16 hours. E. coli JM103 cells were treated with $CaCl_2$ according to a conventional method to obtain a suspension of the E. coli cells in 50 mM $CaCl_2$, and the ligation mixture prepared as above (20 µl) was added to the E. coli suspension to transform the E. coli.

The transformant clones were screened as follows. The suspension containing transformant E.coli cells was diluted in YT soft agar medium contain-

ing X-Gal and IPTG (prepared by adding 10 µl of 10 mM IPTG, 50 µl of 2% X-gal and 0.2 ml of E. coli JM103 suspension grown in a logarithmic growth phase into 3 ml of solution containing 0.6% agar, 0.8% Bacto trypton, 0.5% yeast extract and 0.5% NaCl). 0.3 ml of the diluted suspension was spread on YT agar medium (1.5% agar, 0.8% Bacto trypton, 0.5% yeast extract and 0.5% NaCl), and incubated at 37°C for 16 hours to form plaques. Among the plaques, 10 plaques were selected, and inoculated into 2 x YT liquid medium (1.6% Bacto trypton, 1% yeast extract and 1.0% NaCl) and cultured at 37°C for 8 hours. 1 ml of the cultured medium was centrifuged at 10,000 rpm for 10 minutes to recover a supernatant containing phage. The phage DNA (single strand (DNA) was isolated and purified as follows.

To 800 µl of the phase liquid, 200 µl of 20% polyethylen glycol (PEG 6000) containing 2.5N NaCl was added, and the mixture was allowed to stand at room temperature for 20 minutes and centrifuged at 10,000 rpm for 5 minutes to precipitate the phage. The precipitated phage was dissolved in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and to the solution 50 µl of phenol saturated with water was added, the mixture was vigorously stirred for 5 minutes, and centrifuged at 10,000 rpm for 5 minutes. After sampling 80 µl of the aqueous phase, to the aqueous phase 3 µl of 3M sodium acetate, pH 8.0, and 200 µl of ethanol was added, and the mixture was cooled at -70°C for 10 minutes, and then centrifuged at 10,000 rpm for 10 minutes to precipitate DNA. The precipitated DNA was washed once with ethanol, and dissolved in 50 µl of the above-mentioned TE buffer. A part of a base sequence of each phage DNA was sequenced according to the dideoxy chain termination method (Methods in Enzymology, 65, 560-580, 1980, Academic Press, New York), and among 10 clones, 2 clones containing a lower strand (DNA fragment having a base sequence complementary to mRNA)

were selected. The phage DNA thus obtained were used as a template for in vitro mutation.

(2-a) Incorporation of EcoRI cleavage site and translation initiation codon by in-vitro mutation

To 5 µl of the single strand phage DNA solution described above, 1 µl of 0.2M Tris-HCl buffer, pH 7.5, containing 0.1M $MgCl_2$ and 0.5M NaCl, and 2 µl of water, were added 2 µl of solution containing 10 pmole of 36-mer chemically synthesized DNA fragment (5'-CTCCTAGGTCAGGAATTCATGAATCCCATGTACAAT-3') phosphorylated at the 5' end to form 10 µl of a mixture. The mixture was heated at 65°C for 5 minutes, and allowed to stand for 10 minutes at room temperature.

To the mixture, 1 µl of 0.2 M Tris-HCl buffer (pH7.5) containing 0.1 M MgCl, 2 µl of 0.1 M DTT, 1 µl of 10 mM ATP, 2 µl each of 10 mM dATP, dDTP, dCTP and dTTP, 2 µl of water, 5 units of DNA polymerase I Klenow fragment (Boehringger Manheim) and 2.8 units of T4 DNA ligase (Takara Shuzo) were added, and the mixture was incubated at 15°C for 16 hours. 20 µl of the reaction mixture was used to transform E. coli JM103.

As described above for the preparation of phage DNA, plagues were formed on a YT soft agar medium, and 48 clones were selected. The clones were inoculated to 2 x YT medium, and cultured at 37°C for 8 hours. 1 ml of the cultured medium was centrifuged at 10,000 rpm for 10 minutes to recover the supernatant as phage solution. On the other hand, RF-DNA was extracted and isolated from the precipitated cells according to the alkaline extraction method (Birnboim, H.C. & Doly, J., Nucl. Acid. Res., 7, 1513-1523, 1979). The RF-DNA was then cleaved with 4.2 units of EcoRI (Takara Shuzo) in EcoRI buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$) at 37°C for 1 hour. The reaction mixture was subjected to 2% agarose gel electrophoresis, and a clone providing a DNA fragment of about 530bp was selected. The clone was designated as M13mp8-hANP525.

To 400 ml of 2 X YT liquid medium, 0.4 ml of culture of E. coli JM103 infected with the above-mentioned phage clone and 4 ml of a not-infected culture of E. coil JM103 were inoculated. The medium was then incubated at 37°C for 12 hours. The cultured medium was centrifuged to obtain an infected cell precipitation and a supernatant phage solution. From the infected cells, RF-DNA was obtained by a density-gradient centrifugation method using cesium chloride and ethidium bromide according to a conventional method. On the other hand, from the supernatant phage solution phage DNA was obtained, and the phage DNA was sequenced according to the dideoxy chain termination method. As a result, it was confirmed that a translation initiation codon ATG and Eco RI cleavage recognition site GAATTC, i.e., a base sequence GAATTC ATG, were inserted immediately upstream of the γ-hANP gene.

(2-c) Construction of expression vector for γ-hANP gene (Fig. 6, Fig. 8)

An expression plasmid pS220 was constructed from plasmid pS20 and M13mp8-hANP525. The starting plasmid pS20 was constructed by the inventors, and E. coli N4380/pS20 containing the plasmid pS20 was designated as SBM271, and deposited as FERM BP-535 as described above. The plasmid pS20 contains λ phage P_L promotor, and can express a foreign gene inserted to a site downstream of the promotor under the control of the promotor.

The plasmid pS20 was constructed as follows. Bacteriophage λC1857 DNA was cleaved with BamHI and Hind III to obtain a 2.4 kb DNA fragment containing the λP_L promotor. The DNA fragment was inserted into a region of Hind III-Bam HI in pBR322 to obtain a plasmid which is substantially the same as the plasmid pKO 30 described in Nature 292, 128,1981. To the Hpa I cleavage site of the plasmid thus obtained, 1.3 Kb Hae III DNA fragment containing NutR, tR_1 , CII

and a part of O protein derived from bacteriophage λcy3048 (from Dr. Hiroyuki Shimatake, Medical Department, Toho University) was inserted to obtain plasmid (pS9), wherein CII is present in the direction the same as the transcription direction of λP_L. The plasmid (pS9) was cleaved with Bgl II and Rsa I to obtain a 0.65 Kb DNA fragment containing PL promotor, DNA sequence of protein N' which is a part of an N protein lacking a C terminal, and the Shine-Dalgarno sequence (SD) of a CII gene. The Rsa I and of the 0.65 Kb DNA fragment was added with the Eco RI linker:

-CGGAATTCCG-

-GCCTTAAGGC-

(New England Biolabos, Inc.), and then the Bgl II end of the same DNA fragment was converted to a blunt end with T4 DNA polymerase. The DNA fragment thus obtained was ligated with a DNA fragment prepared by Eco RI cleavage of plasmid pBR322 and conversion of the pBR322 ends to blunt ends to form a plasmid (pS13). In the plasmid (pS13), the P_L promotor is oriented in the direction the same as the transcription direction of the tetracycline resistant gene (Tc^r) derived from pBR322. The plasmid (pS13) was cleaved with Eco RI and Sal I, and a large fragment was isolated. The large fragment was then ligated with a DNA fragment containing a foreign gene, i.e., human γ-interferon gene GIF, which fragment was prepared by cleavage of plasmid pGIF4 with Eco RI and Sal I. The plasmid pGIF4 was disclosed in Japanese Unexamined Patent Publication No. 58-201995 (USP. Serial No. 496176), and E. coli containing the plasmid was designated as SBMG 105 and deposited at the FRA under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure as FERM BP-282 on May 6, 1962.

Starting with the plasmid pS20 and M13mp8-hANP525, the γ-hANP expression vector pS220 was constructed as follows. To 70 µl of Eco RI reaction

buffer (100 mM Tris-Hcl, pH 7.3, 50 mM NaCl, 5 mM $MgCl_2$) containing 8 µg of M13mp8-hANP525 DNA, 15 units of Eco RI and 15 units of Sal I were added, the reaction mixture was incubated at 37°C for 1 hour, and then subjected to the 1.0% agar gel electrophoresis. About 510 bp Eco RI - Sal I DNA fraction containing γ-hANP gene was extracted from the agar gel and purified by the electroelution method described above. On the other hand, to 50 µl of Eco RI reaction buffer containing 2 µg of Plasmid pS20 DNA, 10 units of Eco RI and 10 units of Sal I were added, and the reaction mixture was incubated at 37°C for 1 hour, and then subjected to 1.0% agar gel electrophoresis. According to the above-mentioned electroelution, about 4.3 Kb Eco RI - Sal I DNA fragment containing the λP_L promotor and the SD sequence of the CII gene was obtained. 3 µl of an aqueous solution of the Eco RI - Sal I DNA fragment containing γ-hANP gene and 10 µl of an aqueous solution of the Eco RI - Sal I DNA fragment derived from plasmid pS20 were mixed. To the mixture, 3 µl of ligation buffer with 10 times the concentration described in section (2-a), 3 µl of 100 mM DTT, 3 µl of 1 mM ATP aqueous solution, 6 µl of distillated water, and 2 units of T4 DNA ligase were added, and the mixture was incubated at 16°C overnight to obtain a ligation mixture. The ligation mixture was used to transform E. coli N4830 (from Pharmacia P-L Biochemicals) as follows. The E. coli cells were treated according to a conventional procedure to obtain competent cells. To 300 µl of the treated E. coli cell suspension containing 50 mM $CaCl_2$, 10 µl of the ligation mixture obtained as above was added, and the resulting mixture was allowed to stand for 1 hour in ice. 2.5 µl of L-broth was added to the mixture, which was then incubated at 32°C for 1.5 hours. The resulting mixture was plated on a nutrient agar medium containing 50 µg/ml of ampicillin to select ampicillin resistant colonies which are transformants. The trans-

formants were treated according to a conventional procedure to isolate plasmid DNAs. The plasmids were screened by analysis using restriction enzymes. A plasmid having the γ-hANP gene downstream of the λP_L promotor was selected, and designated as pS220. The transformant containing the plasmid pS220 was designated as E. coil N4380/pS220, and was used in the test for γ-hANP production as described below in detail.

In addition to the γ-hANP gene expression vector pS220, other γ-hANP gene expression vectors were constructed as follows. Plasmid pS20 (Fig. 6) was cleaved with Eco RI, and hydrolyzed with an exonuclease Bal 31 to form DNA fragments having various lengths. The DNA fragments were ligated with Xba I linker (from New England Biolabos Inc.; dCTCTAGAG) to obtain plasmid pS20X. The plasmid pS20X was cleaved with Xba I and Sal I to delete any Xba I - Sal I short fragments. On the other hand, plasmid pIN4GIF54 was cleaved with Xba I and Sal I to obtain a short Xba I - Sal I fragment containing human γ-interferon gene, which short fragment was then inserted to the above-mentioned cleaved pS20X in place of the deleted Xba I - Sal I fragment to form plasmids. Among the plasmids thus formed, a plasmid which can effectively express the human γ-interferon gene when transformed into E. coli was designated as pS83-3. The above-mentioned plasmid pIN4GIF54 and a method for measuring an amount of γ-interferon were disclosed in Japanese Unexamined Patent Publication No. 60-24187 (USP. Serial No. 632204). As shown in Fig. 8, plasmid sP83-3 was cleaved with Eco RI and Sal I to delete the Eco RI - Sal I short fragmemnt consisting of the human γ-interferon gene (GIF). The plasmid M13mp8-hANP525 was cleaved with Eco RI and Sal I to obtain an about 510bp Eco RI - Sal I fragment containing γ-hANP gene, which fragment was then inserted to the cleaved pS83-3 in place of the deleted Eco RI - Sal I fragment consisting of GIF to form plasmid pS223-3. The

plasmid pS223-3 contains an λP_L promotor region, SD sequence of E. coli lpp gene, and γ-hANP gene, in this order. E. coli N4380 transformed with the plasmid pS223-3 was designated as E. coli N4380/pS223-3, and used in the test for γ-hANP production as described below in detail.

Another γ-hANP gene expression vector pS224-3 was constructed as follows. The plasmid pS83-3 was cleaved with Xba I and Eco RI to delete the SD sequence of the lpp gene, and in place of the delected SD sequence of the lpp gene, a chemically synthesized DNA fragment AGGAGGT with Xba I and Eco RI cohesive ends, which is the SD sequence of the bacteriophage MS2A protein gene, was inserted into the cleaved pS83-3 to form plasmid pS84-3. As shown in Fig. 8, the plasmid pS84-3 DNA was cleaved with Eco RI and Sal I to delete the Eco RI - Sal I short fragment consisting of GIF. The plasmid M13mp8-hANP525 DNA was cleaved with Eco RI and Sal I to obtain an about 510bp Eco RI - Sal I fragment containing the γ-hANP gene, which fragment was then inserted into the cleaved pS84-3 DNA in place of the deleted Eco RI - Sal I fragment consisting of GIF to obain plasmid pS224-3. The plasmid pS224-3 thus obtained contained a λp_L promotor region, SD sequence of MS2A, and γ-hANP gene, in this order. E. coli N4380 transformed with the plasmid pS224-3 was designated as E. coli N4380/pS224-3, and tested for the production of γ-hANP.

3) Production of γ-hANP by transformant

Three transformants, i.e., E. coli N4830/pS220, N4830/pS223-3 and N4830/pS224-3, and a control strain E. coli N4830 containing no plasmid were compared. Each of these four strains was cultured in an M9 minimum medium supplemented with 50 μg/ml of amplicillin and 19 natural amino acids except for L-methionene at 32°C until the optical density at 660 nm (OD 660) of the culture reached 0.4. At this point each culture was

divided into two cultures. One of the divided cultures was cultured at the same temperature, i.e., 32°C; and the other was cultured at 42°C. At 15 minutes and 30 minutes after the division of the culture, 0.3 ml of culture was obtained from each culture, and to the culture (0.3 ml), 2 μCi of ^{35}S-methionine with 800 Ci/m mole of specific activity (New England Nuclea) was added. Culturing was continued for 2 minutes and then trichloroacetic acid was added to the concentration of 10% to each culture, which was then cooled to 0°C. The mixture was then centrifuged for 10 minutes, and the precipitate was washed once with ethanol and subjected to the 15% SDS polyacrylamide gel electrophoresis to separate proteins. The gel was dried and subjected to a radioautography to detect bands of proteins labeled with ^{35}S-methionine.

As shown in Fig. 7, for cultures at 42°C of three transformants, i.e., N4830/pS220, N4830/pS223-3, and N4830/pS224-3,a protein band corresponding to a molecular weight of 20Kd was found. The 20Kd band was not found for a culture at 32°C of the three transformants, and a culture at 32°C and 42°C of the control strain. This means that a protein corresponding to the 20Kd band was produced by expression of the γ-hANP gene under the λP_L promotor. The expression product is confirmed to be γ-hANP by the fact that γ-hANP prepared from human atrium cordis migrates to a site the same as the expression product on SDS polyacrylamide gel electrophoresis under the same condition. In the SDS polyacrylamide gel electrophoresis, a standard protein kit for determination of the moleculor weight supplied by Pharmacia P-L Biochemicals was used.

Example 2. Preparaton of γ-hANP from human atrium cordis

377 g of human atrium cordis was removed and boiled in ten volumes of 1 M acetic acid aqueous solution for 10 minutes, to inactivate protease present in the atrium

cordis. The boiled atrium cordis in the acetic acid solution was then cooled to 4°C, added with cooled acetone to two volumes and homogemized with a Polytron homogenizer to extract the γ-hANP. The homogenate thus obtained was centrifuged at 16000 XG for 30 minutes to obtain a supernatant. From the supernatant, acetone was removed by vacuum distillation to obtain 150 ml of an extract containing γ-hANP.

To the extract water was added to 1ℓ of total volume, and the diluted extract was subjected to reverse column chromatography using 90 ml of LCSORB SP-C-ODS column (Chemco). The charged column was washed with 450 ml of 0.1 N aceticacid solution, and eluted with 450 ml of a mixed eluent of water: acetonitrile: 10% trifluoroacetic acid (40:60:1). The elute was fractionated to a green fraction at an early stage and a brown fracton at a later stage. 225 ml of the green fraction was then lyophylized to obtain 90 mg of dried residue.

The residue thus obtained was dissolved in 5 ml of 1 N acetic acid, and the solution was subjected to gel filtration using a Sephadex G-75 column (Pharmacia, 1.8 x 135 cm) equilibrated with a 1 N acetic acid solution. The elution was carried out with 1 N acetic acid, and 5 ml of fractions were obtained. The elution profile is set forth in Fig. 13. Fractions No. 39 to 44 having chicken rectum relaxation activity were combined, and the combined fraction was subjected to HPLC using a TSK ODS column (Toyo Soda, 4.0 x 250 mm) and two eluents, i.e., (A) a mixture of water: acetonitrile: 10% trifluoroacetic acid (90:10:1) and (B) a mixture of water: acetonitrile: 10% acetic acid (40:60:1). The column was equilibrated with eluent (A), and 250 µl of the active fraction obtained from the Sephadex G-75 column was applied to the column. Elution was carried out by linear gradient from (A) to (B) at a flow rate of 1.0 ml/min and at a pressure of 110 to 130 Kg/cm^2 for 80 minutes. The result is set forth in Fig. 14. A peak

fraction having chick rectum relaxation activity (retention time 50 minutes) was obtained, and subjected to HPLC using the same column. Elution was carried out using an eluent (A) water: acetonitrile: 10% trifluoroacetic acid (70:30:1) and an eluent (B) water: acetonitrile: 10% trifluoroacetic acid (40:60:1) in the same manner as described above. The result is set forth in Fig. 15. The main peak was fractionated to obtain about 3.37 nmole of purified γ-hANP for one run. The same procedure was repeated to obtain 404 n mole of γ-hANP.

Example 3. Preparation of parenteral composition

A) Injection solution

Compositon

γ-hANP	2 g
sodium chloride	8 g
ascorbic acid	2 g
sterile water	1 ℓ

Method

γ-hANP and sodium chloride were dissolved in sterile water, an ampule was filled with 5 ml of the solution, and the ampule was then sealed.

B) Lyophilizate

Composition

γ-hANP	2 g
sorbital	20 g

Method

γ-hANP and sorbital were dissolved in 200 ml of sterile water, a vial was filled with 1 ml of the solution, and lyophilized, and the vial was then sealed.

The composition is dissolved in 5 ml of sterile water before parenteral administration.

CLAIMS

1. A DNA fragment comprising a base sequence coding for a peptide occurring in human atrium cordis and having diuretic action or a precursor of the peptide.

2. A DNA fragment according to claim 1, wherein the base sequence codes for a peptide consisting of all or a part of the following amino acid sequence:

Met Ser Ser Phe Ser Thr Thr Thr Val Ser
Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn
Ala Val Ser Asn Ala Asp Leu Met Asp Phe
Lys Asn Leu Leu Asp His Leu Glu Glu Lys
Met Pro Leu Glu Asp Glu Val Val Pro Pro
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala
Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala
Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly
Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu
Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys
Phe Gly Gly Arg Met Asp Arg Ile Gly Ala
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg
Tyr.

3. A DNA fragment according to claim 2, wherein the peptide has the following amino acid sequence:

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala
Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
His Leu Glu Glu Lys Met Pro Leu Glu Asp
Glu Val Val Pro Pro Gln Val Leu Ser Glu
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser
Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly
Ala Leu Gly Arg Gly Pro Trp Asp Ser Ser
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu
Arg Arg Ser Ser Cys Phe Gly Gly Arg Met
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly

Cys Asn Ser Phe Arg Try.

4. A DNA fragment according to claim 2, wherein the base sequence consists of all or a part of the following sequence:

ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC
TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA
GGT CAG ACC AGA GCT AAT CCC ATG TAC AAT
GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC
AAG AAT TTG CTG GAC CAT TTG GAA GAA AAG
ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA
CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG
GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG
CCT CCC TGG ACC GGG GAA GTC AGC CCA GCC
CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC
CCC TGG GAC TCC TCT GAT CGA TCT GCC CTC
CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT
GCC CCT CGG AGC CTG CGG AGA TCC AGC TGC
TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC
CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG
TAC.

5. A DNA fragment according to claim 3, wherein the peptide is coded by the following base sequence:

AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA
GAC CTG ATG GAT TTC AAG AAT TTG CTG GAC
CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT
GAG GTC GTG CCC CCA CAA GTG CTC AGT GAC
CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC
CCC CTC CCT GAG GTG CCT CCC TGG ACC GGG
GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT
GCC CTC GGG CGG GGC CCC TGG GAC TCC TCT
GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG
AGG GCG CTG CTC ACT GCC CCT CGG AGC CTG
CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG
GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC
TGT AAC AGC TTC CGG TAC.

6. A DNA fragment according to any one of the preceding claims, wherein the DNA fragment is

cDNA prepared by using mRNA as a template, which mRNA is obtained from human atrium cordis.

7. A plasmid containing a promotor region, SD sequence, and a DNA fragment comprising a base sequence coding for a peptide occurring in human atrium cordis and having diuretic action or a precursor of the peptide.

8. A plasmid according to claim 7, wherein the peptide consists of all or a part of the following amino acid sequence:

Met Ser Ser Phe Ser Thr Thr Thr Val Ser
Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn
Ala Val Ser Asn Ala Asp Leu Met Asp Phe
Lys Asn Leu Leu Asp His Leu Glu Glu Lys
Met Pro Leu Glu Asp Glu Val Val Pro Pro
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala
Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala
Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly
Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu
Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys
Phe Gly Gly Arg Met Asp Arg Ile Gly Ala
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg
Tyr.

9 A plasmid according to claim 8, wherein the peptide consists of the following amino acid sequence:

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala
Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
His Leu Glu Glu Lys Met Pro Leu Glu Asp
Glu Val Val Pro Pro Gln Val Leu Ser Glu
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser
Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly
Ala Leu Gly Arg Gly Pro Trp Asp Ser Ser
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu

Arg Arg Ser Ser Cys Phe Gly Gly Arg Met
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
Cys Asn Ser Phe Arg Tyr.

10. A plasmid according to claim 8, wherein the amino acid sequence is coded by the following base sequence:

ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC
TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA
GGT CAG ACC AGA GCT AAT CCC ATG TAC AAT
GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC
AAG AAT TTG CTG GAC CAT TTG GAA GAA AAG
ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA
CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG
GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG
CCT CCC TGG ACC GGG GAA GTC AGC CCA GCC
CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC
CCC TGG GAC TCC TCT GAT CGA TCT GCC CTC
CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT
GCC CCT CGG AGC CTG CGG AGA TCC AGC TGC
TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC
CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG
TAC.

11. A plasmid according to claim 9, wherein the amino acid sequence is coded by the following base sequence:

AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA
GAC CTG ATG GAT TTC AAG AAT TTG CTG GAC
CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT
GAG GTC GTG CCC CCA CAA GTG CTC AGT GAC
CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC
CCC CTC CCT GAG GTG CCT CCC TGG ACC GGG
GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT
GCC CTC GGG CGG GGC CCC TGG GAC TCC TCT
GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG
AGG GCG CTG CTC ACT GCC CCT CGG AGC CTG
CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG
GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC

TGT AAC AGC TTC CGG TAC.

12. A plasmid according to any one of claims 7 to 11 wherein the promotor is a λP_L promotor.

13. A plasmid according to any one of claims 7 to 11 wherein the SD sequence is an SD sequence of a CII gene, SD sequence of an lpp gene or SD sequence of an MS2A gene.

14. A plasmid according to claim 7, wherein the plasmid is pS220, pS223-3, or pS224-3.

15. A microorganism transformed with a plasmid containing a promotor region, SD sequence, and a DNA fragment comprising a base sequence coding for a peptide occurring in human atrium cordis and having diuretic action or a precursor of the peptide.

16. A microorganism according to claim 15, wherein the peptide consists of all or a part of the following amino acid sequence:

Met Ser Ser Phe Ser Thr Thr Thr Val Ser
Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn
Ala Val Ser Asn Ala Asp Leu Met Asp Phe
Lys Asn Leu Leu Asp His Leu Glu Glu Lys
Met Pro Leu Glu Asp Glu Val Val Pro Pro
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala
Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala
Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly
Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu
Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys
Phe Gly Gly Arg Met Asp ARg Ile Gly Ala
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg
Tyr.

17. A microorganism according to claim 16, wherein the peptide consists of the following amino acid sequence:

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala
Asp Leu Met Asp Ph Lys Asn Leu Leu Asp

His Leu Glu Glu Lys Met Pro Leu Glu Asp
Glu Val Val Pro Pro Gln Val Leu Ser Glu
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser
Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly
Ala Leu Gly Arg Gly Pro Trp Asp Ser Ser
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu
Arg Arg Ser Ser Cys Phe Gly Gly Arg Met
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
Cys Asn Ser Phe Arg Tyr.

18. A microorganism according to claim 16, wherein the amino acid sequence is coded by the following base sequence:

ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC
TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA
GGT CAG ACC AGA GCT AAT CCC ATG TAC AAT
GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC
AAG AAT TTG CTG GAC CAT TTG GAA GAA AAG
ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA
CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG
GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG
CCT CCC TGG ACC GGG GAA GTC AGC CCA GCC
CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC
CCC TGG GAC TCC TCT GAT CGA TCT GCC CTC
CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT
GCC CCT CGG AGC CTG CGG AGA TCC AGC TGC
TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC
CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG
TAC.

19. A microorganism according to claim 17, wherein the amino acid sequence is coded by the following base sequence:

AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA
GAC CTG ATG GAT TTC AAG AAT TTG CTG GAC
CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT
GAG GTC GTG CCC CCA CAA GTG CTC AGT GAC

CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC
CCC CTC CCT GAG GTG CCT CCC TGG ACC GGG
GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT
GCC CTC GGG CGG GGC CCC TGG GAC TCC TCT
GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG
AGG GCG CTG CTC ACT GCC CCT CGG AGC CTG
CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG
GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC
TGT AAC AGC TTC CGG TAC.

20. A microorganism according to any one of claims 15 to 19 wherein the microorganism is E. coli.

21. A process for production of a peptide occurring in human atrium cordis and having diuretic action and/or a precursor of the peptide, comprising culturing a microorganism transformed with a plasmid containing a promotor region, SD sequence, and a DNA fragment comprising a base sequence coding for the peptide, or a precursor peptide to form the respective peptide and recovering the same; or

converting such a peptide or precursor to an acid addition salt thereof; or

converting an acid addition salt of such a peptide or precursor to the free base form thereof.

22. A process according to claim 21, wherein the peptide has all or part of the following amino acid sequence:

Met Ser Ser Phe Ser Thr Thr Thr Val Ser
Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
Gly Gln Thr Arg Ala Asn Pro Met Try Asn
Ala Val Ser Asn Ala Asp Leu Met Asp Phe
Lys Asn Leu Leu Asp His Leu Glu Glu Lys
Met Pro Leu Glu Asp Glu Val Val Pro Pro
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala
Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala
Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly

Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu
Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys
Phe Gly Gly Arg Met Asp Arg Ile Gly Ala
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg
Tyr.

23. A process according to claim 22, wherein the peptide has the following amino acid sequence:

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala
Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
His Leu Glu Glu Lys Met Pro Leu Glu Asp
Glu Val Val Pro Pro Gln Val Leu Ser Glu
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser
Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly
Ala Leu Gly Arg Gly Pro Trp Asp Ser Ser
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu
Arg Arg Ser Ser Cys Phe Gly gly Arg Met
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
Cys Asn Ser Phe Arg Tyr.

24. A process according to claim 21 wherein the DNA fragment is as set forth in any of claims 2 to 6.

25. A precursor of a diuretic peptide γ-hANP of the following amino acid sequence:

Met Ser Ser Phe Ser Thr Thr Thr Val Ser
Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn
Ala Val Ser Asn Ala Asp Leu Met Asp Phe
Lys Asn Leu Leu Asp His Leu Glu Glu Lys
Met Pro Leu Glu Asp Glu Val Val Pro Pro
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala
Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala
Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly

Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu
Leu Lys Ser Lys L u Arg Ala Leu Leu Thr
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys
Phe Gly Gly Arg Met Asp Arg Ile Gly Ala
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg
Tyr,

and acid addition salt thereof.

26. A diuretic peptide γ-hANP of the following amino acid sequence:

Asn Pro Met Tyr Asn Ala Val Ser Asn Ala
Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
His Leu Glu Glu Lys Met Pro Leu Glu Asp
Glu Val Val Pro Pro Gln Val Leu Ser Glu
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser
Pro Leu Pro Glu Val Pro Pro Trp Thr Gly
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly
Ala Leu Gly Arg Gly Pro Trp Asp Ser Ser
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu
Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu
Arg Arg Ser Ser Cys Phe Gly Gly Arg Met
Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly
Cys Asn Ser Phe Arg Tyr,

and acid addition salt thereof.

27. A diuretic composition containing a peptide γ-hANP according to claim 26 or an acid addition salt thereof with a conventional pharmaceutical additive.

28. A hypotensor composition containing a peptide γ-hANP according to claim 26, or an acid addition salt thereof with a conventional pharmaceutical additive.

29. A composition according to claim 27 or claim 28, wherein the composition is a solution for parenteral administration and the additive is a buffer, an osmotic pressure adjusting agent or a preservative, or a combination thereof.

30. A composition according to any one of claims 27, 28 or 29 wherein the composition isa solution for parenteral administration and contains about 0.000005 to 5% of the γ-hANP.

31. A composition according to any one of claims 27 to 30, wherein the composition is in a lyophylized form.

32. The medical use of γ-hANP as defined in claim 26, or an acid addition salt thereof, or a composition according to any one of claims 27 to 31.

Fig. 1

```
                                                  10                                                  20
Asn Pro Met Tyr Asn Ala Val Ser Asn Ala Asp Leu Met Asp Phe Lys Asn Leu Leu Asp

                                                  30                                                  40
His Leu Glu Glu Lys Met Pro Leu Glu Asp Glu Val Val Pro Pro Gln Val Leu Ser Glu

                                                  50                                                  60
Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser Pro Leu Pro Glu Val Pro Pro Trp Thr Gly

                                                  70                                                  80
Glu Val Ser Pro Ala Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly Pro Try Asp Ser Ser

                                                  90                                                 100
Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr Ala Pro Arg Ser Leu

                                                 110                                                 120
Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly

                                126
Cys Asn Ser Phe Arg Tyr
```


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Fig. 2

```
 1                                                  10                                                  20
Met Ser Ser Phe Ser Thr Thr Thr Val Ser Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu

                                                    30                                                  40
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn Ala Val Ser Asn Ala Asp Leu Met Asp Phe

                                                    50                                                  60
Lys Asn Leu Leu Asp His Leu Glu Glu Lys Met Pro Leu Glu Asp Glu Val Val Pro Pro

                                                    70                                                  80
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser Pro Leu Pro Glu Val

                                                    90                                                  100
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly

                                                    110                                                 120
Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr

                                                    130                                                 140
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala

                                                    150
Cln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr
```


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Fig. 3

```
                                                  10                                                  20
AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC AAG AAT TTG CTG GAC

                                                  30                                                  40
CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA CAA GTG CTC AGT GAG

                                                  50                                                  60
CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG CCT CCC TGG ACC GGG

                                                  70                                                  80
GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC CCC TGG GAC TCC TCT

                                                  90                                                 100
GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT GCC CCT CGG AGC CTG

                                                 110                                                 120
CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC CAG AGC GGA CTG GGC

                        126
TGT AAC AGC TTC CGG TAC
```


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Fig. 4

```
 1                                                  10                                                  20
ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA

                                                    30                                                  40
GGT CAG ACC AGA GCT AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC

                                                    50                                                  60
AAG AAT TTG CTG GAC CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA

                                                    70                                                  80
CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG

                                                    90                                                  100
CCT CCC TGG ACC GGG GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC

                                                    110                                                 120
CCC TGG GAC TCC TCT GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT

                                                    130                                                 140
GCC CCT CGG AGC CTG CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC

                                                    150
CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG TAC
```


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Fig. 5

Fig. 5A

Fig. 5B

Fig. 5A

```
                                                                    -90
                                                                    5'-ACAGACGTAGG  -80

CCAAGAGAGGGGAACCAGAGAGGAACCAGAGGGGAGAGACAGAGCAGCAAGCAGTGGATTGCTCCTTGACGACGCCAGC   -1

 1                                        10                                  20
Met Ser Ser Phe Ser Thr Thr Thr Val Ser Phe Leu Leu Leu Leu Ala Phe Gln Leu Leu
ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA   60

                                         30                                  40
Gly Gln Thr Arg Ala Asn Pro Met Tyr Asn Ala Val Ser Asn Ala Asp Leu Met Asp Phe
GGT CAG ACC AGA GCT AAT CCC ATG TAC AAT GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC  120

                                         50                                  60
Lys Asn Leu Leu Asp His Leu Glu Glu Lys Met Pro Leu Glu Asp Glu Val Val Pro Pro
AAG AAT TTG CTG GAC CAT TTG GAA GAA AAG ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA  180

                                         70                                  80
Gln Val Leu Ser Glu Pro Asn Glu Glu Ala Gly Ala Ala Leu Ser Pro Leu Pro Glu Val
CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC CCC CTC CCT GAG GTG  240
```


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Fig.5B

```
                                        90                                  100
Pro Pro Trp Thr Gly Glu Val Ser Pro Ala Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly
CCT CCC TGG ACC GGG GAA GTC AGC CCA GCC CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC  300

                                       110                                  120
Pro Trp Asp Ser Ser Asp Arg Ser Ala Leu Leu Lys Ser Lys Leu Arg Ala Leu Leu Thr
CCC TGG GAC TCC TCT GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT  360

                                       130                                  140
Ala Pro Arg Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Met Asp Arg Ile Gly Ala
GCC CCT CGG AGC CTG CGG AGA TCC AGC TGC TTC GGG GGC AGG ATG GAC AGG ATT GGA GCC  420

                                       150
Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Try
CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG TAC TGA AGATAACAGCCAGGGAGGACAAGCAGGGCTG  487

GGCCTAGGGACAGACTGCAAGAGGCTCCTGTCCCCTGGGGTCTCTGCTGCATTTGTGTCATCTTGTTGCCATGGAGTTG  566

TGATCATCCCATCTAAGCTGCAGCTTCCTGTCAACACTTCTCACATCTTATGCTAACTGTAGATAAAGTGGTTTGATGG  645

TGACTTCCTCGCCTCTCCCACCCCATGCATTAAATTTTAAGGTAGAACCTCACCTGTTACTGAAAGTGGTTTGAAAGTG  724

AATAAACTTCAGCACCATGGACAGAAGAC-3'                                               753
```

Fig. 6

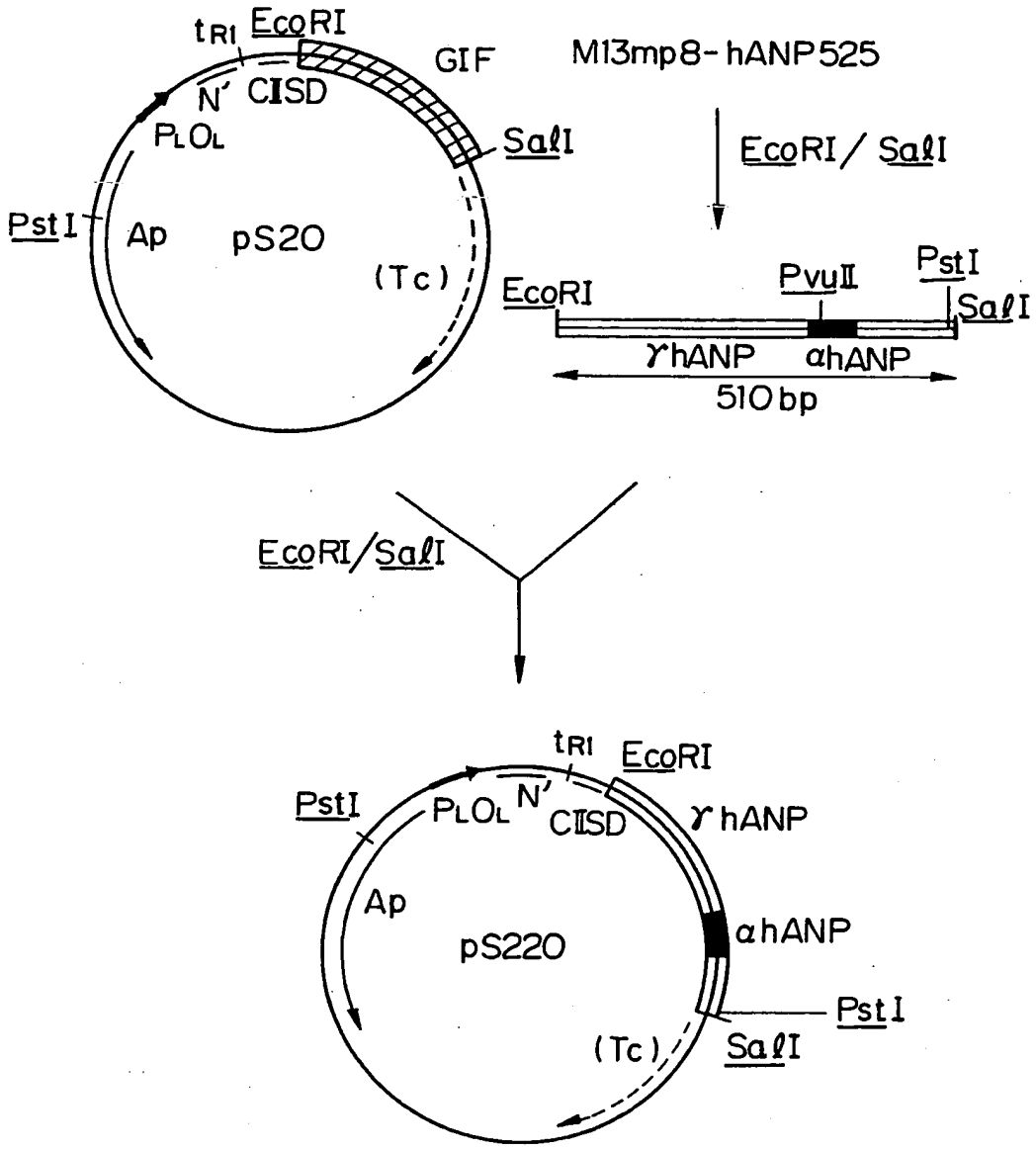
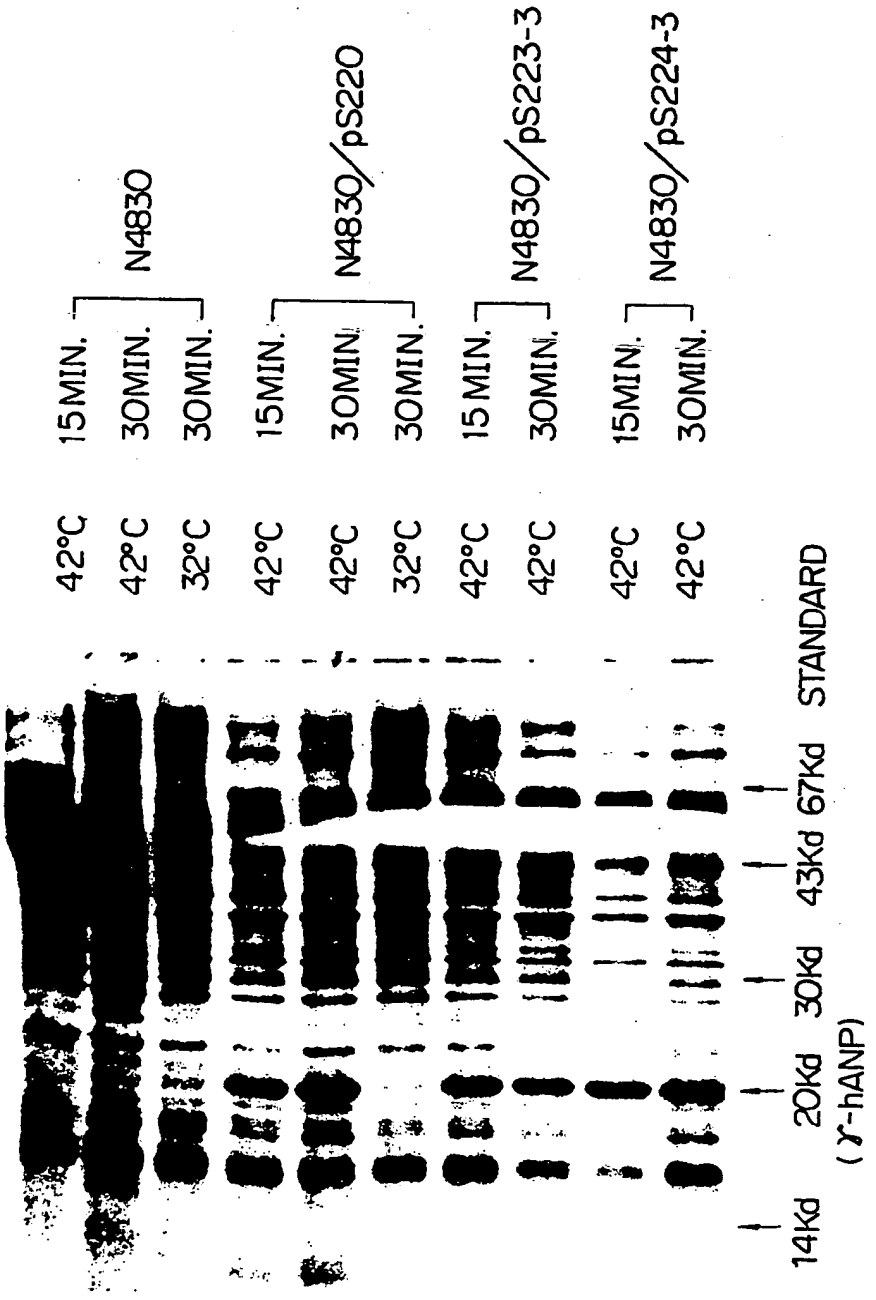

tRI
EcoRI
GIF
N'
CIISD
PLOL
SalI
PstI
Ap
pS20
(Tc)
M13mp8-hANP525
EcoRI / SalI
EcoRI
PvuII
PstI
SalI
γhANP
αhANP
510bp
EcoRI/SalI
tRI
EcoRI
PstI
PLOL
N'
CIISD
γhANP
Ap
pS220
αhANP
PstI
(Tc)
SalI

Fig. 7


42°C
42°C
32°C
42°C
42°C
32°C
42°C
42°C
42°C
42°C
15MIN.
30MIN.
30MIN.
15MIN.
30MIN.
30MIN.
15 MIN.
30MIN.
15MIN.
30MIN.
N4830
N4830/pS220
N4830/pS223-3
N4830/pS224-3
14Kd
20Kd
30Kd
43Kd
67Kd
STANDARD
(γ-hANP)

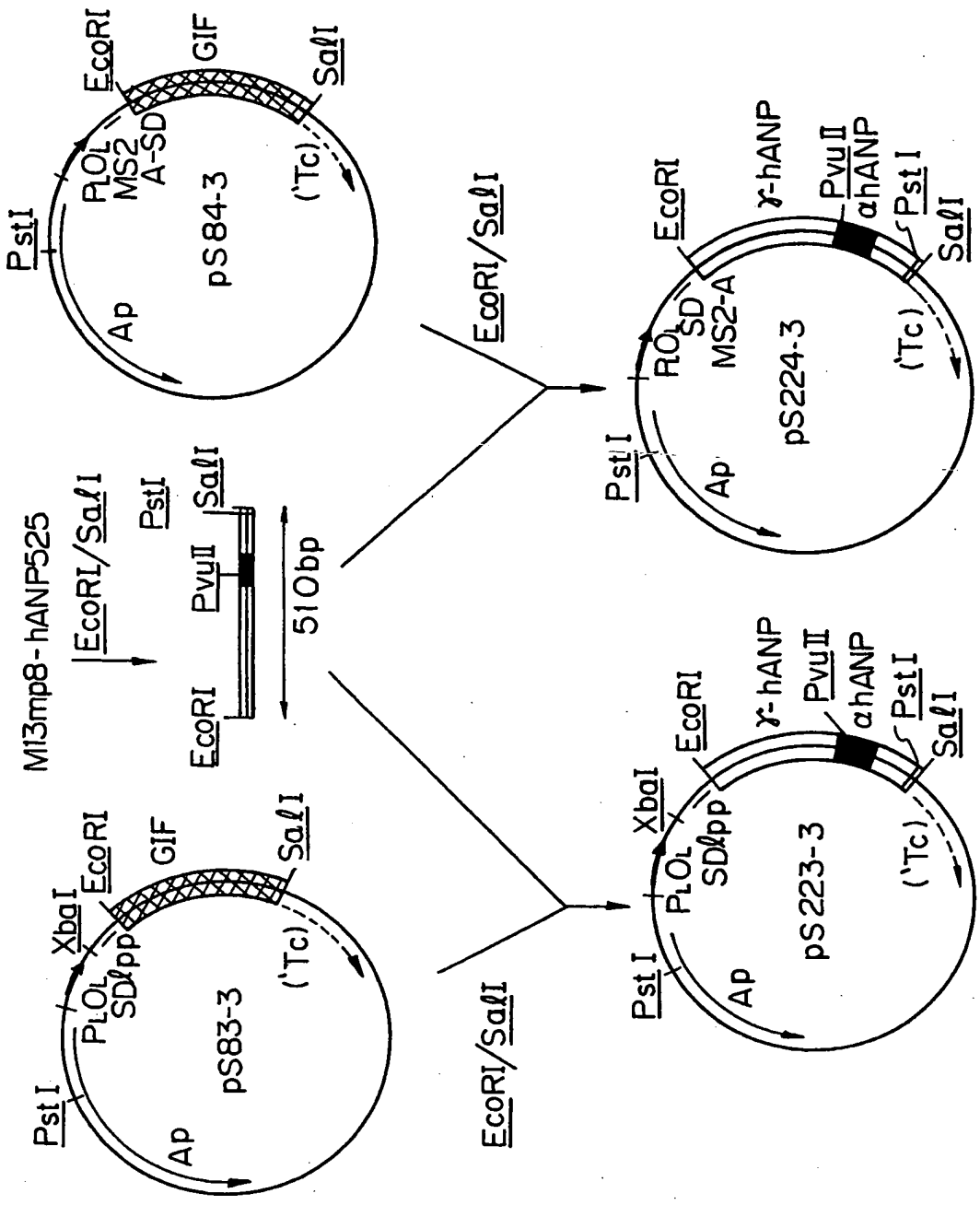

M13mp8-hANP525
EcoRI/SalI
PstI
SalI
PvuII
EcoRI
510bp
EcoRI/SalI
EcoRI/SalI
PstI
EcoRI
GIF
SalI
PLOL
MS2
A-SD
pS84-3
Ap
('Tc)
XbaI
SDlpp
pS83-3
EcoRI
RO
SD
MS2-A
pS224-3
γ-hANP
PvuII
αhANP
PstI
SalI
XbaI
pS223-3

Fig. 8

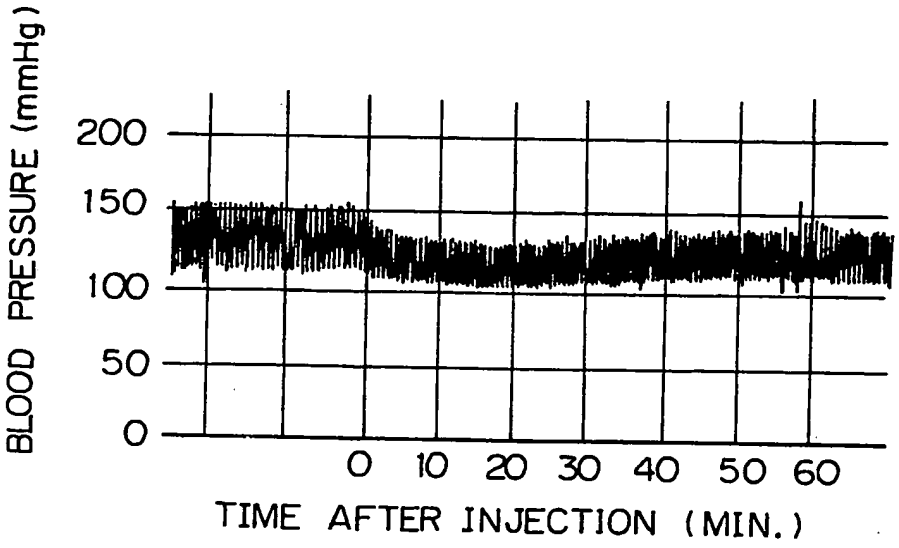
Fig. 9

Met Asp Arg Ile Gly

3'- TAC CT(G/A) GC(T/G/C/A) TAG(T/A) CC-5' PROBE I

3'- TAC CT(G/A) TC(T/C) TAG(T/A) CC-5' PROBE II

Fig. 12



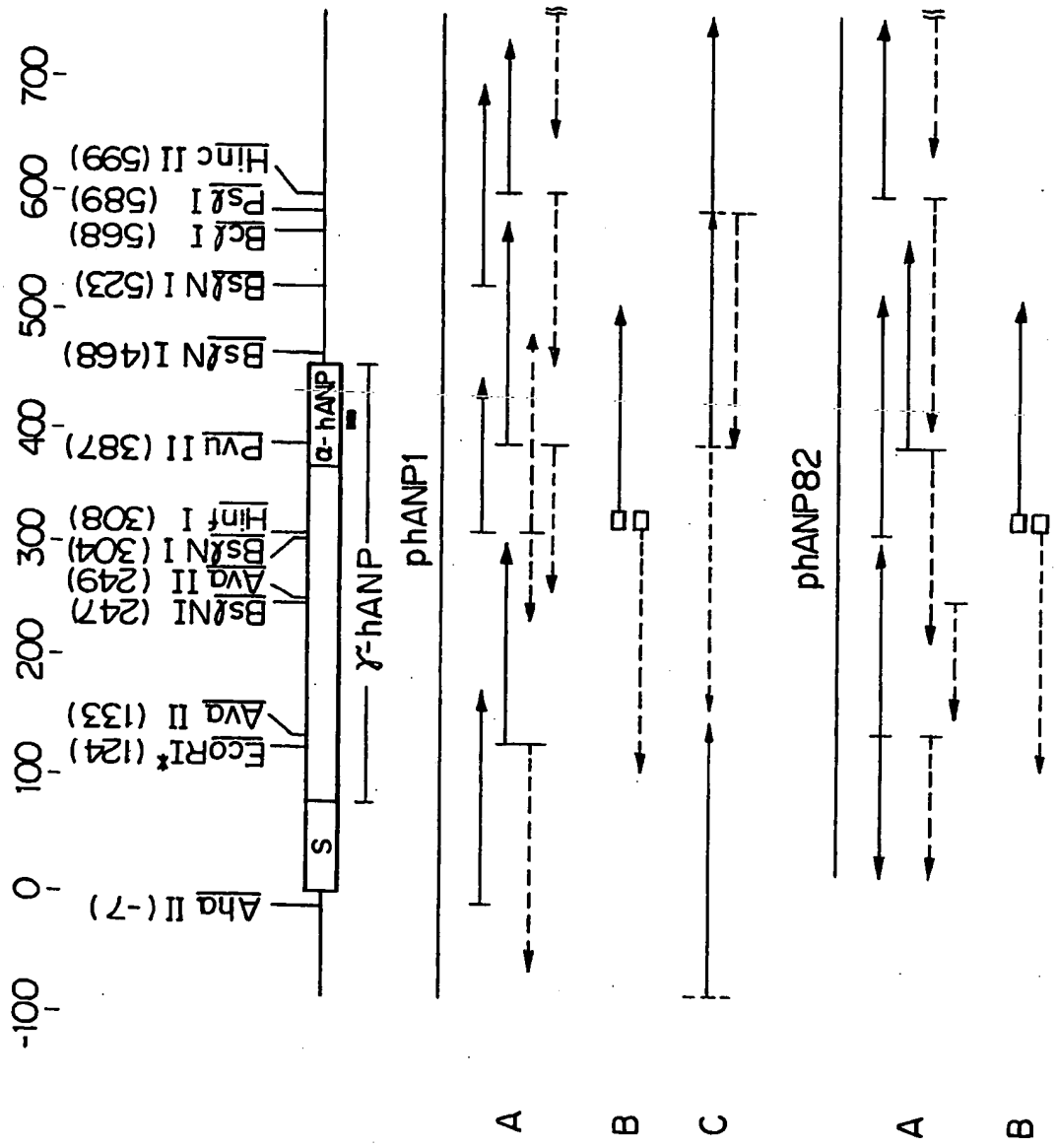

-100
0
100
200
300
400
500
600
700
Aha II (-7)
EcoRI* (124)
Ava II (133)
BsℓNI (247)
Ava II (249)
BsℓN I (304)
Hinf I (308)
Pvu II (387)
BsℓN I(468)
BsℓN I (523)
Bcℓ I (568)
Psℓ I (589)
Hinc II (599)
S
α-hANP
γ-hANP
phANP1
phANP82
A
B
C
A
B

Fig. 10

Fig. 11

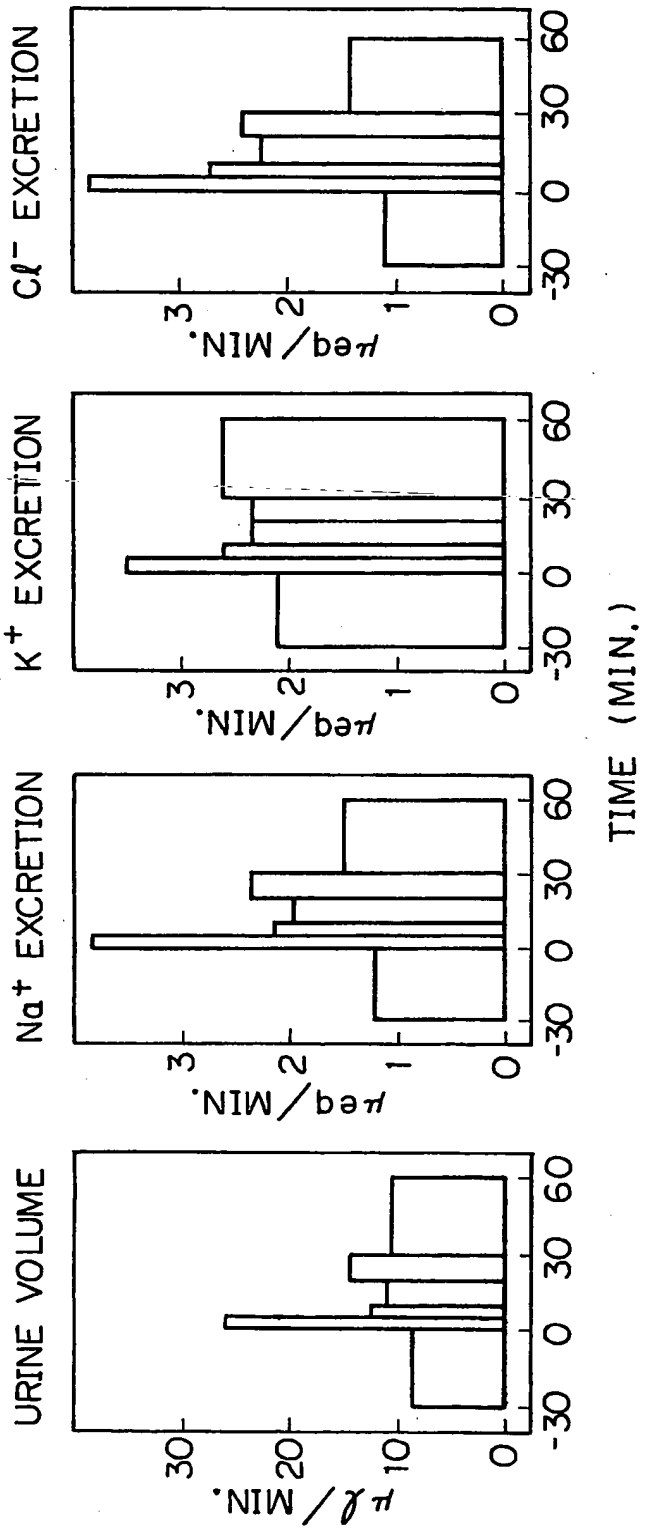
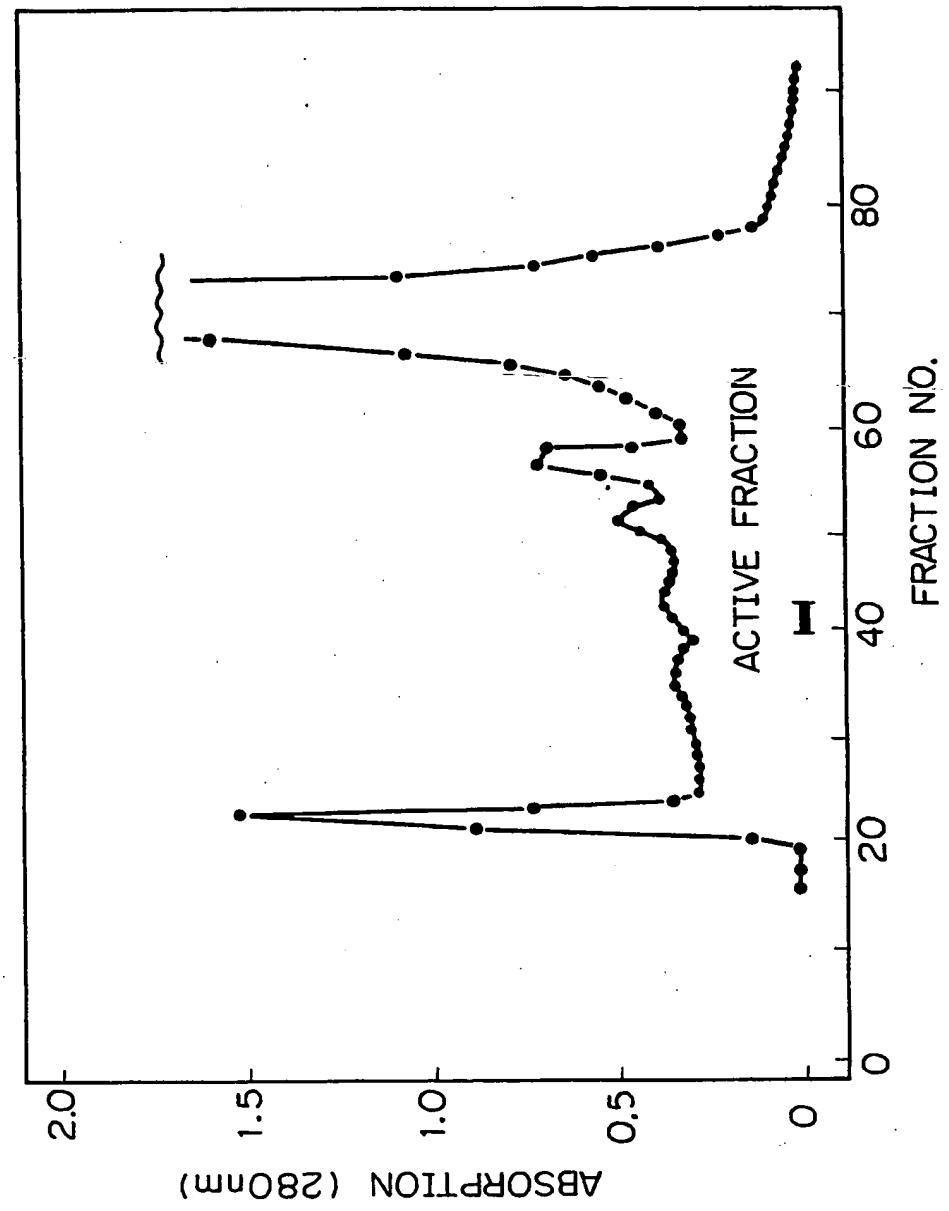

Cl⁻ EXCRETION
K⁺ EXCRETION
Na⁺ EXCRETION
URINE VOLUME
μeq / MIN.
μℓ / MIN.
0
1
2
3
10
20
30
-30
0
30
60
TIME (MIN.)

Fig. 13


ABSORPTION (280nm)
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1.5
1.0
0.5
0
0
20
40
60
80
FRACTION NO.
ACTIVE FRACTION

Fig. 14

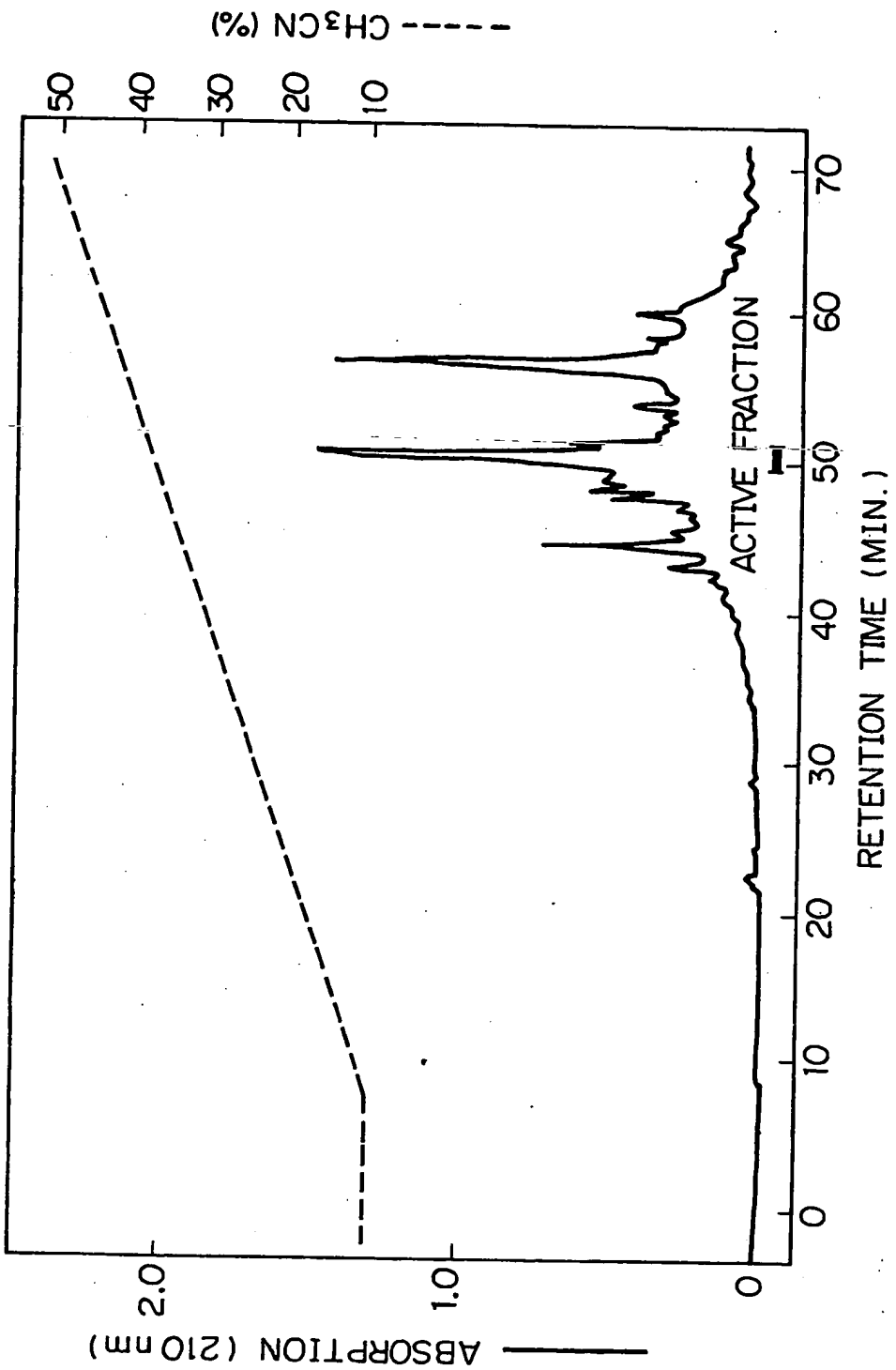
ABSORPTION (210 nm)
2.0
1.0
0
RETENTION TIME (MIN.)
0
10
20
30
40
50
60
70
ACTIVE FRACTION
---- CH3CN (%)
50
40
30
20
10

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Fig. 15